

DEVELOPMENT & IMPLEMENTATION OF THE PHYTOTEST PROJECT

Siobhán Kavanagh¹, Claire Brennan¹, Josephine Lyons², Tara Chamberlain³, Rafael Salas², Siobhán Moran², Joe Silke² and Majella Maher¹

¹DNA lab, National Diagnostics Centre, NUI, Galway, Co. Galway.

²Phytoplankton Unit, MEFS, Marine Institute, Rinville, Oranmore, Co. Galway.

³Biotoxin Unit, MEFS, Marine Institute, Bantry, Co. Cork.

Introduction

The Irish shellfish industry, worth approximately €60 million to the economy in 2006 (Browne *et al.*, 2007), is adversely affected by the presence of harmful microalgae such as *Dinophysis* and *Pseudo-nitzschia* species. Several *Dinophysis* species have been shown to produce okadaic acid and dinophysistoxins (DTXs), which are associated with Diarrhetic Shellfish Poisoning (DSP). The DSP toxin producing species *D. acuta* and *D. acuminata* occur in Irish coastal waters throughout the year, primarily from late Spring through to early Autumn, and the majority of closures of Irish mussel-farms during the summer months are attributed to their presence. Members of the *Pseudo-nitzschia* genus are the causative agents of Amnesic Shellfish Poisoning (ASP) in scallops. In 2005, an extended toxicity period caused by ASP and DSP resulted in prolonged closures at many sites. The first closures due to ASP toxicity in rope mussels occurred in that year.

In order to satisfy EU legislative requirements pertaining to the production and export of shellfish (EC Regulations, No. 853/2004 and No. 854/2004, which replaced the EU Shellfish Hygiene Directive 91/492/EEC in January 2006), the Irish Marine Institute (MI) have put a programme in place to monitor the presence of harmful algal species and biotoxins in coastal waters. This monitoring programme currently relies on microscopic identification of phytoplankton species and biochemical analysis of shellfish tissue for toxins. Microscopic identification of phytoplankton species is time consuming and requires a high level of expertise (Penna *et al.*, 2007). While *Dinophysis* may be identified to species level by a trained taxonomist using light microscopy, this is not the case for *Pseudo-nitzschia* species. Intensive electron microscopy investigation is required for species identification and this technique cannot be easily integrated into a routine monitoring programme.

Molecular techniques utilise unique sequence signatures within genomes for identification and discrimination between closely related species. Molecular identification can be performed on a variety of platforms and therefore provides a rapid alternative to laborious morphological investigation. Nucleic acid-based diagnostic assays have been developed and applied to the identification and quantification of toxic phytoplankton species (Scholin *et al.*, 1997; Saito *et al.*, 2002; Galluzzi *et al.*, 2004). Real-time PCR based assays have been developed to detect and/or quantify species including *Alexandrium*, *Pfiesteria*, *Heterosigma*, *Lingulodinium* and *Chattonella* (Bowers *et al.*, 2000; Hosoi-Tanabe & Sako, 2005; Moorthi *et al.*, 2005; Coyne *et al.*, 2005).

Phytotest is a 3-year research and development project funded through the Marine Institute Strategic Research Programme in Advanced Technologies as part of the National Development plan 2000-2006. The project is a collaboration between the National Diagnostics Centre at NUI Galway and the MI and involves the development of real-time PCR assays for *Dinophysis* and *Pseudo-nitzschia* species that are important in Irish waters. In the current final phase of the project, the real-time PCR assays are being transferred to the MI to support the phytoplankton monitoring service.

The potential for real-time PCR assays in HAB detection

What potential do these real-time PCR assays have for the detection of HAB species? *Dinophysis* can be identified to species level using light microscopy. However the morphology of many *Dinophysis* species can be variable (Edvardsen *et al.*, 2003) and extensive taxonomic experience is required for accurate species identification. The examination of samples is time-consuming when a large amount of biomass is present, limiting sample through-put. Conversely real-time PCR is automated, rapid and allows for a high-throughput of samples. A real-time PCR assay for detection and differentiation of the key Irish *Dinophysis* species, *D. acuta* and *D. acuminata*, is useful as a research tool, enabling the analysis of multiple spatial or temporal samples.

The availability of real-time PCR assays for the identification of *Pseudo-nitzschia* to species level, in particular key toxic species such as *P. australis*, adds significant value to the monitoring of HABs. Currently, light microscopy classifies *Pseudo-nitzschia* species into two groups, the *delicatissima* group or the *seriata* group, based on size. In the event of the occurrence of high numbers of *Pseudo-nitzschia* cells in a sample, a number of questions arise. Is it a unialgal bloom? Are toxic or non-toxic species present? Real-time PCR assays provide a rapid alternative to Electron Microscopy (EM) with the capability to answer these questions. Additionally, if required, sequencing of the PCR product generated in the assay can be performed to confirm species identity.

Methodology*Development of real-time PCR assays*

The first stage in the development of our assay method was to obtain single cells (SCs) of target species from wild samples and to set up phytoplankton cultures. During the course of the project, *Pseudo-nitzschia* (*P. australis*, *P. fraudulenta*, *P. delicatissima* and *P. pungens*) and indigenous phytoplankton species cultures were maintained at the MI for sequence generation and assay testing. *Dinophysis* species cannot be readily cultured so isolation of SCs from preserved phytoplankton samples was required to obtain sequence information for indigenous *D. acuta* and *D. acuminata* cells.

Sequence information was generated for selected targets from indigenous *D. acuta* and *D. acuminata* and for *Pseudo-nitzschia* species established in culture. Analysis of the sequence information identified the large ribosomal subunit (LSU) in *Dinophysis* species and the internally transcribed spacer regions (ITS) in *Pseudo-nitzschia* species as the most suitable regions for the design of PCR primers and probes (Figure 1). For Phytotest, the real-time PCR assays incorporating these primers and DNA hybridization probes were designed to be run on the LightCycler®. A pair of fluorescently labelled probes hybridizes to single-stranded DNA from the target region as it is generated in the PCR reaction. When the two probes hybridize in close proximity to each other, energy is transferred between them and emitted in a process known as Fluorescence Resonance Energy Transfer (FRET). This energy is proportional to the increasing amount of PCR product generated and is read by the real-time PCR instrument (Anon, 2000). By combining hybridization probe technology with an analysis function of the LC® software, melt peak analysis, it is possible to generate species-specific melt peaks, which can distinguish target species from non-target species within a sample. In Phytotest, the real-time PCR assays were designed to operate under a common PCR protocol, providing the potential to identify a range of different *Dinophysis* and *Pseudo-nitzschia* species in the same analytical run.

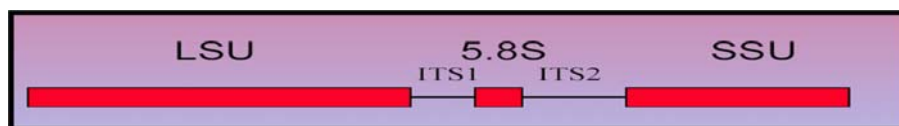


Figure 1. The large ribosomal subunit (LSU) and internal transcribed spacer (ITS) regions of ribosomal DNA gene.

The selection or development of a DNA extraction method is another critical step in the assay method. The primary requirement of a DNA extraction method is that all species present within the sample are represented within the final DNA extract. Initial sample processing involves filtration of 25 ml samples onto a 1 µm cellulose nitrate filter to catch the maximum biomass present within the sample. Samples may be processed immediately or stored preserved with Lugol's iodine for at least 1 year, enabling the method to be applied to study archive samples.

The release of nucleic acids from cells is another important step in the DNA extraction procedure. To ensure detection of target cells that are present in low numbers, it is also crucial to obtain the purest DNA possible for real-time PCR, free from protein, salt and humic acid contaminants. *Dinophysis* cells are easily broken open but *Pseudo-nitzschia* cells possess a hard silica frustule so a physical pre-extraction step, freezing of cells with liquid nitrogen and thawing to 80 °C, is required. In Phytotest, DNA extraction was performed using a chemical extraction procedure.

Following real-time PCR assay design and optimisation of the nucleic acid extraction method, evaluation of the assays is performed initially using SCs or DNA extracts from pure cultures and plasmids containing the target (LSU or ITS) regions. Assay parameters including specificity and sensitivity are determined. Evaluation of wild samples containing target species is performed, to determine the detection capabilities of the assays using real samples with a background microbial and eukaryotic community.

Results

Dinophysis species real-time PCR assay

Initial evaluation of the *Dinophysis* species real-time PCR assay involved testing with SCs of *D. acuta* and *D. acuminata* and with plasmids incorporating the target regions from both *Dinophysis* species. Melt peak analysis of the probes consistently yielded a melt peak at 61⁰C for SCs of *D. acuta* isolated from different Irish coastal locations and for plasmids containing the assay target region for *D. acuta* (Figure 2). Melt peak analysis of the *D. acuminata* SCs with the probes produced melt-peaks at 48⁰C, approximately 13⁰C lower than for *D. acuta*. This melt peak temperature for *D. acuminata* was confirmed when a plasmid containing the target region for *D. acuminata* was included in the real-time PCR assay (Figure 2).

The next step in the assay evaluation process involved testing of wild samples containing *Dinophysis* cells. Samples from the MI phytoplankton-monitoring programme were selected for testing, as they are routinely examined by light microscopy for the presence of HAB species. Fifty-five Lugols iodine preserved samples from the 2006 MI phytoplankton-monitoring programme were tested with the assay. Thirty-three of these samples were reported to contain *Dinophysis* species cells based on microscopic analysis, with cell numbers in the range of 1-8 in 25 ml. Twelve samples contained only 1 cell of either *D. acuta* or *D. acuminata*. The real-time PCR assay detected the presence of *Dinophysis* species in all samples reported to contain *Dinophysis* cells. The real-time PCR assay identified *D. acuta* or *D. acuminata* in DNA extracts from all samples reported to contain either species by microscopy. The percentage occurrence of *Dinophysis* species within these samples is illustrated in Figure 3.

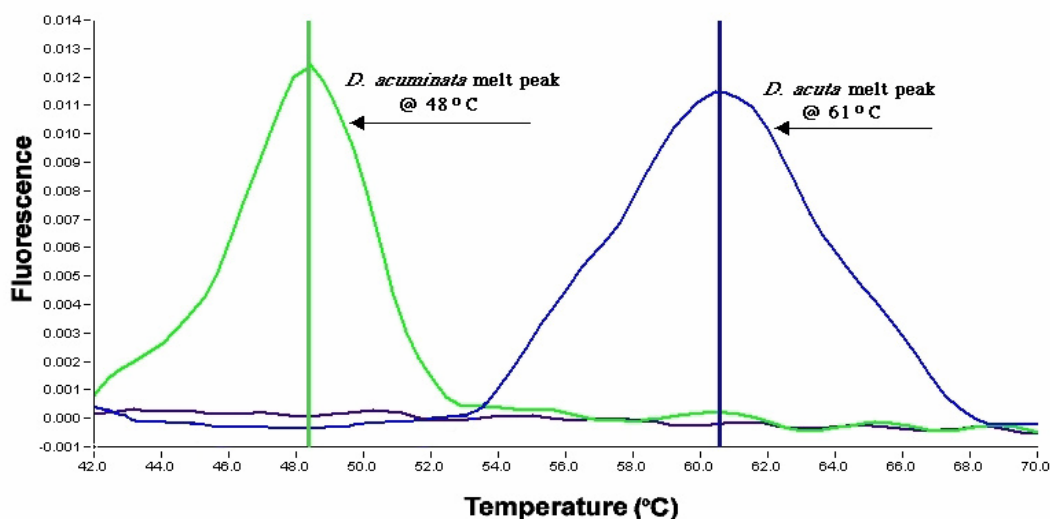


Figure 2. Real-time PCR assay for the detection of *D. acuta* and *D. acuminata* using Hybridization Probes. Both species are simultaneously identified with the assay.

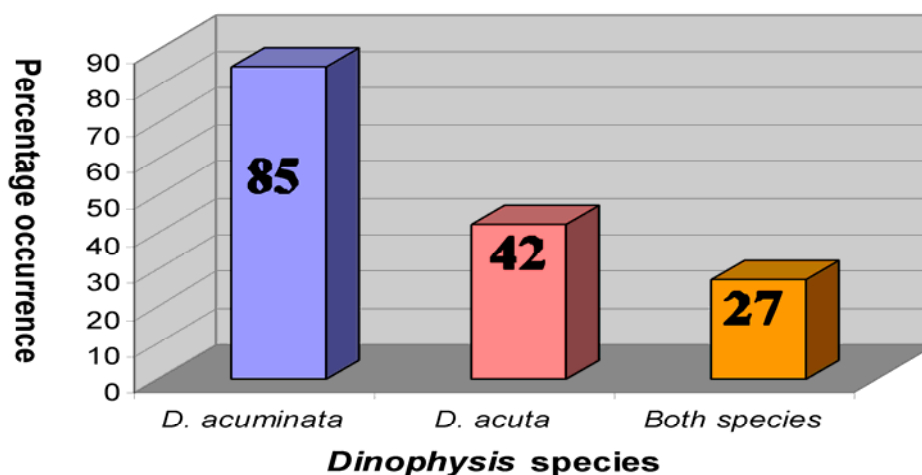


Figure 3. Percentage occurrence of *Dinophysis* species in 2006 MI phytoplankton monitoring programme samples.

An advantage of hybridization probe technology is the potential to detect and distinguish between two or more species with a single probe set, using melt-peak analysis. Specific-melt peaks were observed for *D. acuta* and *D. acuminata* with the real-time PCR assay for four samples reported to contain both species by light microscopy, illustrating that this assay simultaneously detects and discriminates between species, even when occurring at low numbers. There was no detection of *Dinophysis* by the assay in the MI samples where *Dinophysis* cells were not observed by light microscopy. A specificity panel, including a range of phytoplankton species commonly found in Irish waters (Table 1) was tested against the assay and no cross-reactivity was observed. Phytotest successfully developed a real-time PCR assay capable of identifying the two important *Dinophysis* species in Irish waters when present at 1 cell/25ml.

Table 1. Phytoplankton specificity panel tested with the real-time PCR assays.

<i>Phytoplankton species</i>	
<i>Pseudo-nitzschia australis</i> ^a	<i>Fragilariopsis</i> cf. species ^b
<i>Pseudo-nitzschia fraudulenta</i> ^a	<i>Gymnodinium</i> cf. species ^a
<i>Pseudo-nitzschia delicatissima</i> ^a	<i>Lingulodinium polyedrum</i> ^a
<i>Pseudo-nitzschia pungens</i> ^a	<i>Leptocylindrus danicus</i> ^b
<i>Pseudo-nitzschia multiseriata</i> ^a	<i>Licmophora</i> cf. species ^b
<i>Pseudo-nitzschia calliantha</i> ^a	<i>Myrionecta rubra</i> ^a
<i>Pseudo-nitzschia seriata</i> ^a	<i>Navicula</i> cf. <i>erifuga</i> ^b
<i>Dinophysis acuta</i> ^a	<i>Nitzschia lecontei</i> ^b
<i>Dinophysis acuminata</i> ^a	<i>Pleurosigma</i> cf. species ^b
<i>Phalacroma rotundata</i> ^a	<i>Prorocentrum dentatum</i> ^a
<i>Alexandrium minimum</i> ^a	<i>Prorocentrum lima</i> ^a
<i>Alexandrium tamarense</i> ^a	<i>Prorocentrum micans</i> ^a
<i>Alexandrium fundyense</i> ^a	<i>Prorocentrum minimum</i> ^a
<i>Alexandrium catenella</i> ^a	<i>Protoperidinium brevipes</i> ^b
<i>Akashiwo sanguinea</i> ^a	<i>Prorocentrum reticulatum</i> ^b
<i>Asterionellopsis glacialis</i> ^b	<i>Rhizosolenia fragilissima</i> ^b
<i>Ceratulina pelagica</i> ^b	<i>Scripsiella</i> cf. species ^b
<i>Chaetoceros debilis</i> ^b	<i>Skeletonema costatum</i> ^b
<i>Chaetoceros</i> cf. species ^a	<i>Skeletonema marinoi</i> ^b
<i>Coscinodiscus wailesii</i> ^a	<i>Striatella unipunctata</i> ^b
<i>Cylindrotheca closterium</i> ^b	<i>Teleaulax</i> species ^a
<i>Cyclotella meneghiniana</i> ^b	<i>Thalassiosira rotula</i> ^b
<i>Dinobryon pellucidum</i> ^b	<i>Tintinnid</i> cf. species ^b
<i>Ditylum brightwellii</i> ^b	

^a denotes phytoplankton species that were directly tested with the real-time PCR assays as single cell templates or DNA extracts of pure cultures

^b denotes phytoplankton species that were present in MI phytoplankton monitoring programme samples

Pseudo-nitzschia species real-time PCR assays

For initial evaluation of the *Pseudo-nitzschia* species real-time PCR assays, DNA extracts from pure cultures of *Pseudo-nitzschia* species and plasmids incorporating the target regions were tested. Evaluation of the *P. australis* assay, determined a species-specific melt peak at 56-57 °C for *P. australis* (Figure 4a). The *P. fraudulenta* assay produced a species-specific melt peak at 55 °C for *P. fraudulenta* (Figure 4b) and the *P. delicatissima* assay produced a species-specific melt peak at 58 °C for *P. delicatissima* (Figure 4c). Evaluation of the *P. pungens* assay determined a species-specific melt peak at 60 °C for *P. pungens* (Figure 4d). The *P. pungens* real-time PCR assay also detected and identified other *Pseudo-nitzschia* species including *P. australis* with a melt peak at 49 °C and *P. fraudulenta* with a small melt peak at 42 °C. In addition, *P. multiseriata* produces a melt peak at 53 °C with this assay (Figure 4e). All *Pseudo-nitzschia* species assays were tested for specificity using a panel of *Pseudo-nitzschia* species and indigenous phytoplankton species (Table 1). No cross reactivity was observed for any of the assays with non-target species.

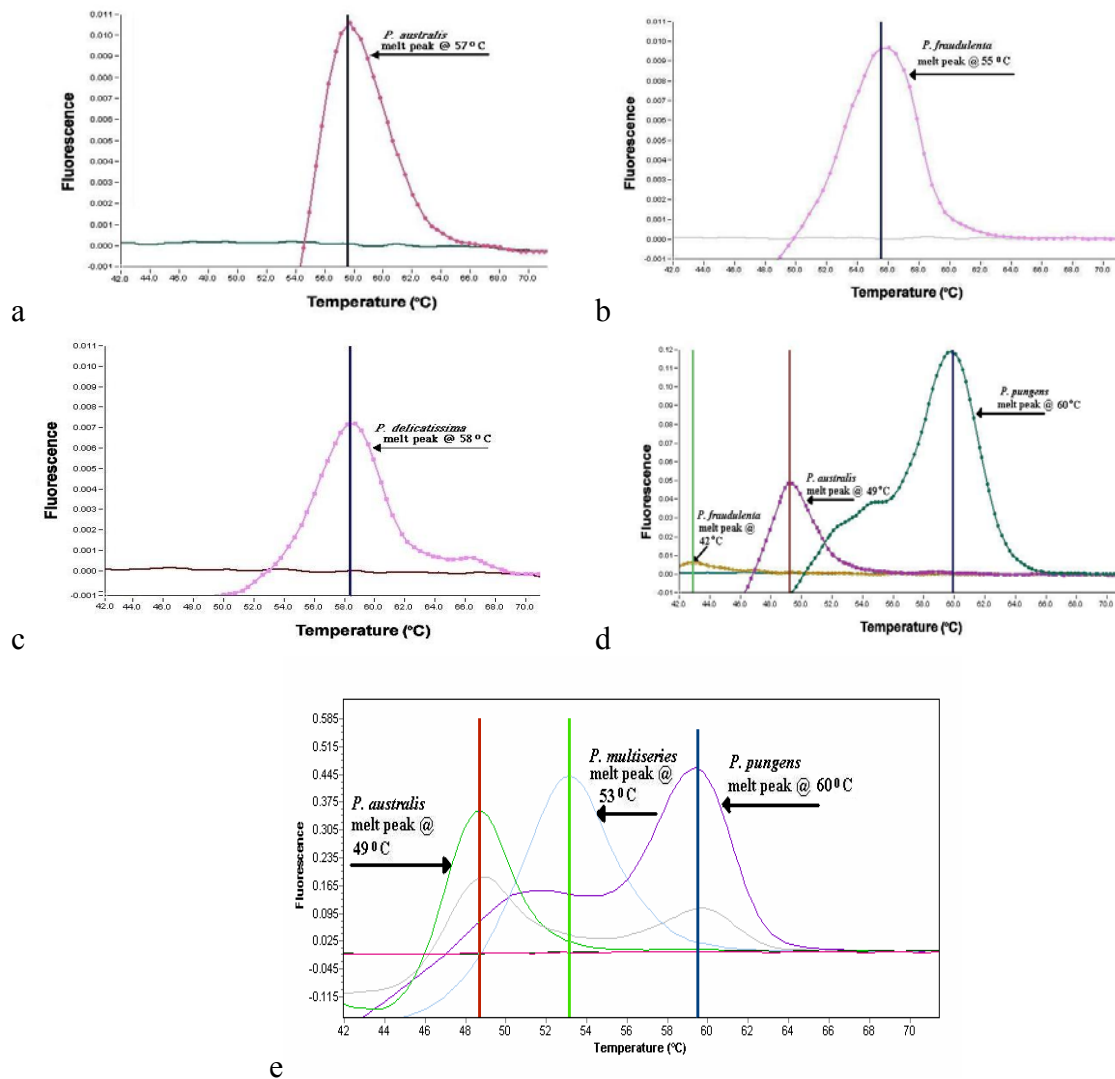


Figure 4a-e. Real-time PCR assays designed for the detection of *Pseudo-nitzschia* species.
a. The *P. australis* assay yields a melt peak at 56-57 °C with *P. australis*.
b. The *P. fraudulenta* assay yields a melt peak at 55 °C with *P. fraudulenta*.
c. The *P. delicatissima* assay yields a melt peak at 58 °C with *P. delicatissima*.
d. The *P. pungens* assay yields a melt peak at 60 °C with *P. pungens*. Melt peaks are also detected at 49 °C with *P. australis* and at 42 °C with *P. fraudulenta*.
e. Melt peak analysis *P. pungens* assay probes yields a peak at 53°C with *P. multiseriis*.

Evaluation of the *Pseudo-nitzschia* species assays was performed using wild samples that had been examined for the presence of *Pseudo-nitzschia* cells by light microscopy. Fifty-eight Lugols iodine preserved samples from the 2006 MI phytoplankton-monitoring programme were tested with each of the *Pseudo-nitzschia* species (*P. australis*, *P. fraudulenta*, *P. delicatissima*, *P. pungens*) real-time PCR assays. Forty-one of the samples contained *Pseudo-nitzschia* cells, with numbers ranging from 40-3889 cells in 25 ml preserved sample. In addition to these samples, seven 2006 Lugols iodine preserved samples were received for testing from Dunstaffnage Marine laboratory. All seven samples contained *Pseudo-nitzschia* cells, with numbers varying from 31-2750 cells in 25 ml.

All of the 2006 samples reported to contain *Pseudo-nitzschia* cells produced melt peaks with at least one of the *Pseudo-nitzschia* species real-time PCR assays. The assays identified the presence of two or more species in 78% of the samples, supporting the reported co-occurrence

of *Pseudo-nitzschia* species from the literature (Hasle *et al.*, 1996, Cusack *et al.*, 2004). Figure 5 illustrates typical real-time PCR assay results obtained for a MI phytoplankton-monitoring programme sample. Melt peaks, indicating the presence of *P. australis*, *P. fraudulenta*, *P. delicatissima*, *P. multiseriis* or *P. pungens*, were absent from the seventeen samples where *Pseudo-nitzschia* cells were not observed by light microscopy.

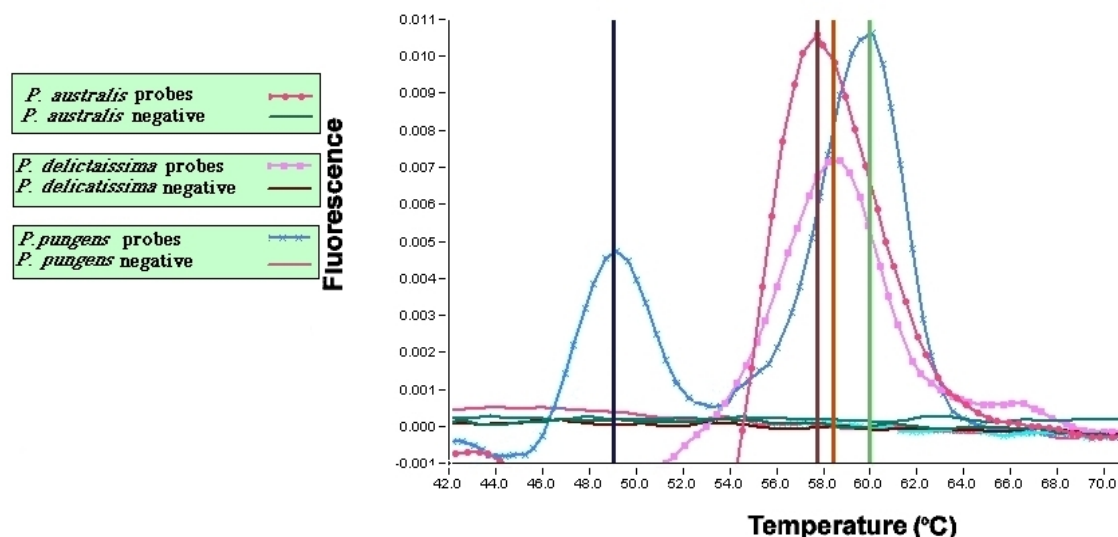


Figure 5. Real-time PCR assays results from a DNA extract of the 2006 Hawks Nest, Mannin Bay sample. This sample was reported to contain 140,760 *Pseudo-nitzschia* cells/L by light microscopy. Three *Pseudo-nitzschia* species were detected within the sample

In addition to the 2006 samples, a DNA extract of 173 cells isolated from a preserved seawater sample was tested with all of the assays. This seawater sample was taken from Castlemaine Harbour as part of the phytoplankton-monitoring programme and was associated with a toxic bloom event in April 2005. The *P. australis* real-time PCR assay detected the presence of *P. australis* in the DNA extract. This result was further confirmed by sequencing of the PCR product and Transmission Electron Microscopy (TEM).

Implementation of Phytotest

Since July 2007, the technology transfer phase of the project has begun with the purchase and installation of the LC[®] 480 instrument. Training of MI Phytoplankton Unit staff in real-time PCR and nucleic acid extraction methodologies has commenced. Initial testing of the assays with positive controls indicated that the real-time PCR assays for *Dinophysis* and *Pseudo-nitzschia* species are working successfully on the LC[®] 480 instrument. Currently, the specificity and limits of detection for these assays are being verified. A performance evaluation of the assays for the identification of the relevant toxic species in wild samples is being performed between the MI (LC[®] 480 instrument) and at the NDC (LC[®] 2.0 capillary machine) as part of a validation of the real-time PCR assays. It is expected that the real-time PCR assays will be used by the MI Phytoplankton unit to support the monitoring programme after March 2008.

Acknowledgements

Thanks to the Dunstaffnage Marine laboratory, Oban, Argyll, Scotland and the EU SPIES Project, NUI Galway for the provision of wild samples and phytoplankton culture material for testing. This project (Grant-aid Agreement No. AT/04/02/02) is carried out with the support of the Marine Institute and the Marine RTDI Measure, Productive Sector Operational Programme, National Development Plan 2000-2006.

References

- Anon, 2000. LightCycler operator's manual. Mannheim: Roche Molecular Biochemicals.
- Bowers, H. A., Tengs, T., Glasgow, Jr., H. B., Burkholder, J.A. M., Rublee, P. A. and Oldach, D. W., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Applied and Environmental Microbiology*, 66: 4641-4648.
- Browne, R., Deegan, B., O'Carroll, T., Norman, M. and Ó'Cinnéide, M., 2007. Status of Irish Aquaculture, 2006. Marine Institute/Bord Iascaigh Mhara/Taighde Mara Teo: 113pp.
- Coyne, K. J., Handy, S. M., Demir, E., Whereat, E. B., Hutrchins, D. A., Portune, K. J., Doblin, M. A. and Cary, S. C., 2005. Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnology and Oceanography-Methods*, 3: 381-391.
- Cusack, C., Raine, R. and Patching, J. W., 2004. Occurrence of species from the genus *Pseudo-nitzschia* Peragallo in Irish waters. *Biology and Environment: Proceedings of the Royal Irish Academy*, 104B, 1: 55-74.
- Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K. S., Medlin, L. K., Dahl, E., Brubak, S. and Paasche, E., 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analysis of rDNA. *Journal of Phycology*, 39: 395-408.
- Galluzzi L., Penna, A., Bertozzini, E., Vila, M., Garcés, E. and Magnani, M., 2004. Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a Dinoflagellate). *Applied and Environmental Microbiology*, 70: 1199-1206.
- Hasle, G.R., Lange, C.B. and Syvertsen, E.E, 1996. A review of *Pseudo-nitzschia*, with special reference to the Skagerrak, North Atlantic and adjacent waters. *Helgoländer Meeresuntersuchungen*, 50: 131-75.
- Hosoi-Tanabe, S. and Sako, Y., 2005. Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by real-time PCR. *Marine Biotechnology*, 7: 506-514.
- Moorthi, S. D., Countway, P. D., Stauffer, B. A. and Caron, D. A., 2005. Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. *Microbial. Ecology*, 52: 136-150.
- Penna, A., Bertozzini, E., Battocchi, C., Galluzzi, L., Giacobbe, M. G., Vila, M., Garces, E., Lugliè, A. and Magnani, M., 2007. Monitoring of HAB species in the Mediterranean Sea through molecular methods. *Journal of Plankton Research*, 29: 19-38.
- Saito, K., Drgon, T., Robledo, J. A. F., Krupatkina, D. N. and Vasta, G. R., 2002. Characterization of the rRNA locus of *Pfiesteria piscicida* and the development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. *Applied and Environmental Microbiology*, 68: 5393-5407.
- Scholin, C., Miller, P., Buck, K. and Chavez, F., 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using rRNA-targeted probes. *Limnology and Oceanography*, 42: 1265-1272.